DNA Fingerprinting via AFLP of *Polyporus squamosus* Huds.:Fr.

An Honors Thesis (HONRS 499)

by

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Table of Contents

Abstract:

In this experiment, DNA from the mushroom *Polyporus squamosus* was fingerprinted using the technique amplified fragment length polymorphism (AFLP). The morphologic appearance of many fungi can vary a lot within a species, causing discontinuity over where species boundaries fall. *P. squamosus* is a species that has a well-defined appearance and morphology. The DNA fingerprints of this unambiguous species will help shed light on the relationship between genomic content, genomic diversity, and outward appearance.

Introduction:

AFLP stands for amplified restriction length polymorphism, and is a molecular biology technique used to create reliable DNA fingerprints. The components needed are:

- DNA to be fingerprinted
- Restriction endonuclease enzymes
- Short DNA molecules called primers
- DNA polymerase enzyme, which generates new DNA from a DNA template
- Label for DNA (fluorescence or radioactivity)

I will first outline the theory behind the techniques and tools used in this process, the most complex of which is polymerase chain reaction. I will also discuss relevant information on DNA, which should aid the reader when reading the explanations of the work performed, and give helpful background information. The procedures following this are general descriptions, meant to give the reader understanding of the concepts and how they work in the research process. After all of the science background, I will discuss the importance of this research, and reflect on my experiences. Following this will be my paper on the research, using the standard format of the Journal of Biological Chemistry.

On DNA:

DNA (deoxyribonucleic acid): the molecule used to transmit genetic information, which codes for every protein produced in a cell. It is a double stranded molecule, with the four bases (adenine, guanine, thymine, and cytosine) encoding the genetic information. The bases of each strand face each other, and are internal in the double stranded molecule. The backbone of DNA is composed of alternating sugar and phosphate groups, and protects the bases. The two strands are linked via hydrogen bonds between the bases. Hydrogen bonds are not true bonds but weak attraction forces, which allow the DNA strands to come apart easily for replication and other cellular processes where the code of bases needs to be accessed. Adenine forms hydrogen bonds with thymine, so adenine and thymine form a base pair. The same is true with guanine and cytosine. DNA is a directional molecule, with two non-unique ends, designated 5′ (five prime) and 3′. The DNA sequence (of bases) is read starting at the 5′ end and reading towards the 3′ end. The two strands of DNA run in opposite directions (antiparallel)- see Figure 2.

Figure 1.

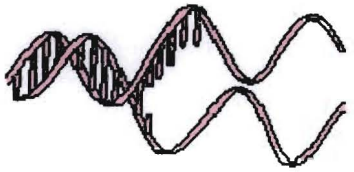

Figure 1. DNA is shown in its normal double- stranded state, and when denatured.

Figure 2.

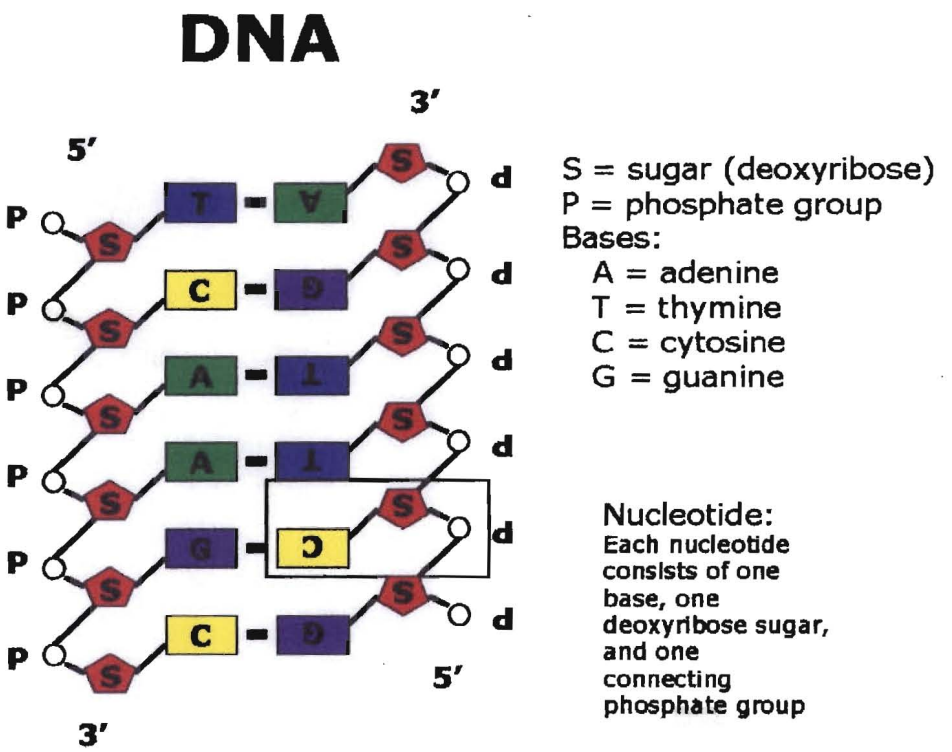

Figure 2. The structure of DNA. The backbone of DNA consists of alternating deoxyribose sugars and phosphate groups. The bases are connected to each sugar. The strands are also antiparallel, with the 3′ end of one strand aligning with the 5′ end of the other. Base pairing must follow the A-T and G-C pairs. Each strand is made up of many nucleotide building blocks.

Overview of research procedures and concepts:

Extraction of DNA from sample:

The first step of performing a DNA extraction is to break through the cell membrane and/or cell wall. For fungi, both are present. Like plants, fungi have a tough wall composed of polysaccharides, which gives support and protection. This wall must be broken without damaging the DNA inside it, which is no easy task. The most effective way is to freeze the tissue with liquid nitrogen, which is at -198° Celsius (-320° Fahrenheit), and grind the tissue to a fine powder with a mortar and pestle.

The next step is to precipitate the unwanted cellular molecules, like lipids and proteins, which are very abundant in the cell. Detergents are used to do this. The ground tissue is then added to a solution containing special detergents, and centrifuged. Centrifugation will pellet the debris at the bottom, and the DNA will be left in the liquid (supernatant), which is transferred to a new tube.

Now, the DNA is out of the cell, but must be concentrated and purified from this solution to be useful in research. The properties of DNA make it associate with water molecules in the cell. When DNA is put into a solution containing high concentrations of certain salts called chaotropic salts, it will form a different shape, excluding water. When this dehydrated DNA is in the presence of silica, it will bind to it. In this way, the DNA is purified: it will bind to a silica column when exposed to chaotropic salts, and can be rinsed of any contaminants. This allows any other molecules that may have carried over from the previous steps to be washed away. When the dehydrated DNA is exposed to water, its stronger attractions for water will make it break its bonds to silica. The DNA is now purified, and is in a useful concentration for further techniques. The DNA is then frozen at -20°C, the normal temperature of a household freezer, and can be stored for many years before it begins to degrade.

AFLP procedure:

A specific amount of DNA [100 nanograms (10^{-9} grams)] is used in the procedure. First of all, the sample's DNA is exposed to restriction endonuclease enzymes. These enzymes were discovered in bacteria, and their original purpose is to digest foreign DNA (like a virus) that may have entered the bacterial cell. They are used frequently in molecular biology because they cut the DNA backbone at very precise points in the sequence. For example, the restriction endonuclease EcoRI (so named because it was the first restriction enzyme isolated from *E. coli*) recognizes this sequence:
5′ GAATTC 3′

It cuts the sequence at the arrows, between the bases adenine and guanine for each strand:

Figure 3.

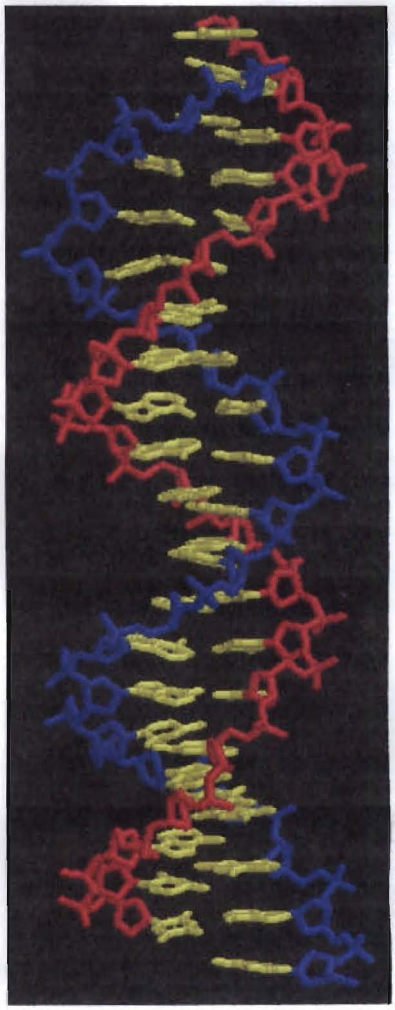

Figure 3. The structure of DNA. One strand is colored blue, the other red. The bases connecting the two strands are in yellow, and are not physically bonded together, as the hydrogen bonds connecting them are not true bonds, just attraction forces. The 3′ end of the blue strand is at the top.

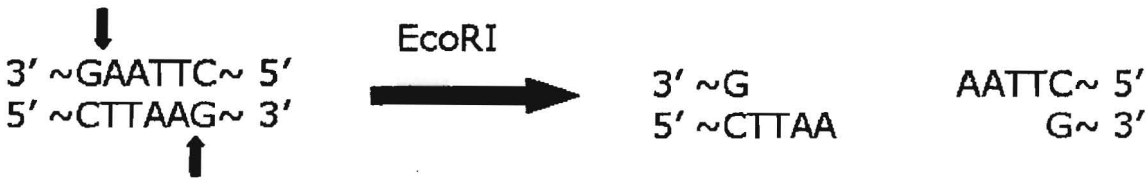

Figure 4. The action of the enzyme EcoRI cuts DNA at a specific sequence.

It leaves what are called 'sticky ends' on the new pieces of DNA it created: one strand of the double-stranded DNA molecule has a few extra bases (TTAA or AATT) than the other strand.

The other restriction enzyme used, MseI, also leaves sticky ends:

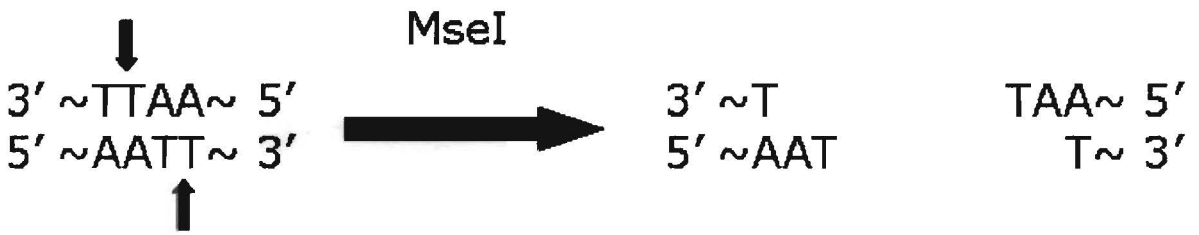

Figure 5. The enzyme MseI cuts DNA at a specific sequence.

The known sequences of the restriction endonucleases allow us to employ another molecular biology technique called PCR. PCR stands for polymerase chain reaction, and is a way of generating DNA using real cellular molecules and enzymes, but in a test tube instead of inside a cell. We use this technique after the sample DNA has been digested with restriction endonucleases.

PCR (polymerase chain reaction) (part of AFLP procedure):

The first step in PCR is to separate the strands of the template (original) DNA. This is done by raising the temperature to around 94°C (almost the temperature at which water boils, 100°C), which will make the two strands of DNA come apart. The increased temperature makes the weak attractions between the bases of the DNA dissociate, so the bases will be exposed:

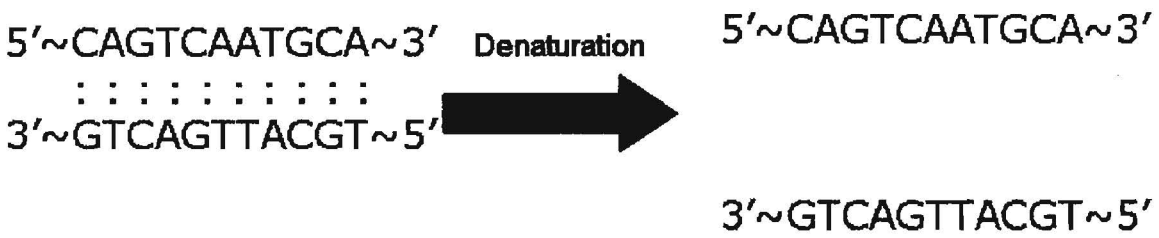

Figure 5. The denaturation of the double-stranded DNA molecule leaves two single-stranded molecules with the bases exposed.

Once the bases are exposed, short DNA molecules commonly called primers are able to base pair with the DNA to be copied (target DNA):

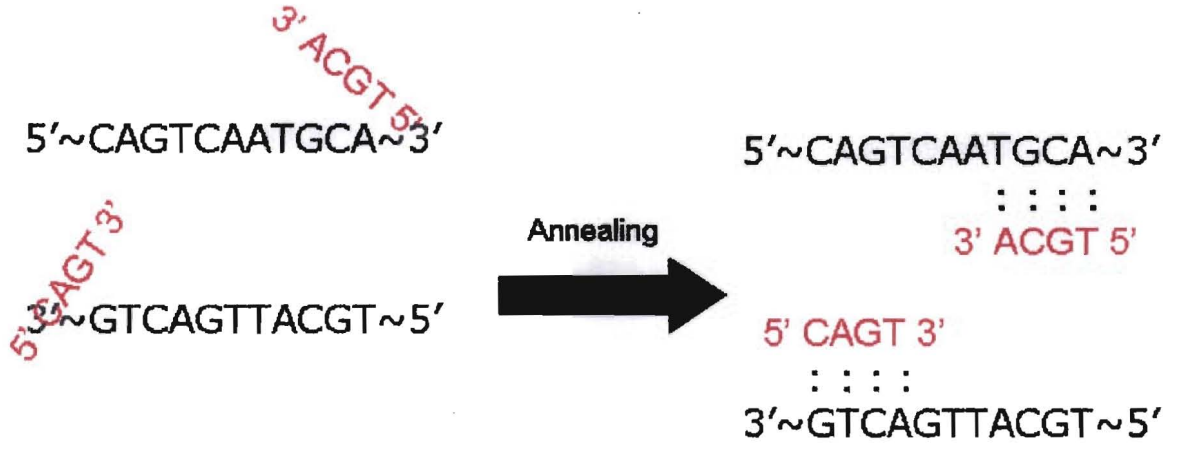

Figure 6. After denaturation takes place, the temperature is lowered quickly, which makes it very difficult for the long strands of DNA to re-anneal, allowing the small DNA molecules known as primers to anneal to the region they can base pair with.

The annealing of the primers depends on the sequence of the primers required; the broad range of temperatures used is 45- 65°C, and this step lasts 30- 45 seconds.

Primers are required for the next step, where an enzyme called DNA polymerase creates new DNA between the primers using free nucleotides. The enzyme requires a short fragment of DNA with a free 3' end to synthesize new DNA, both in cells and in the test tube.

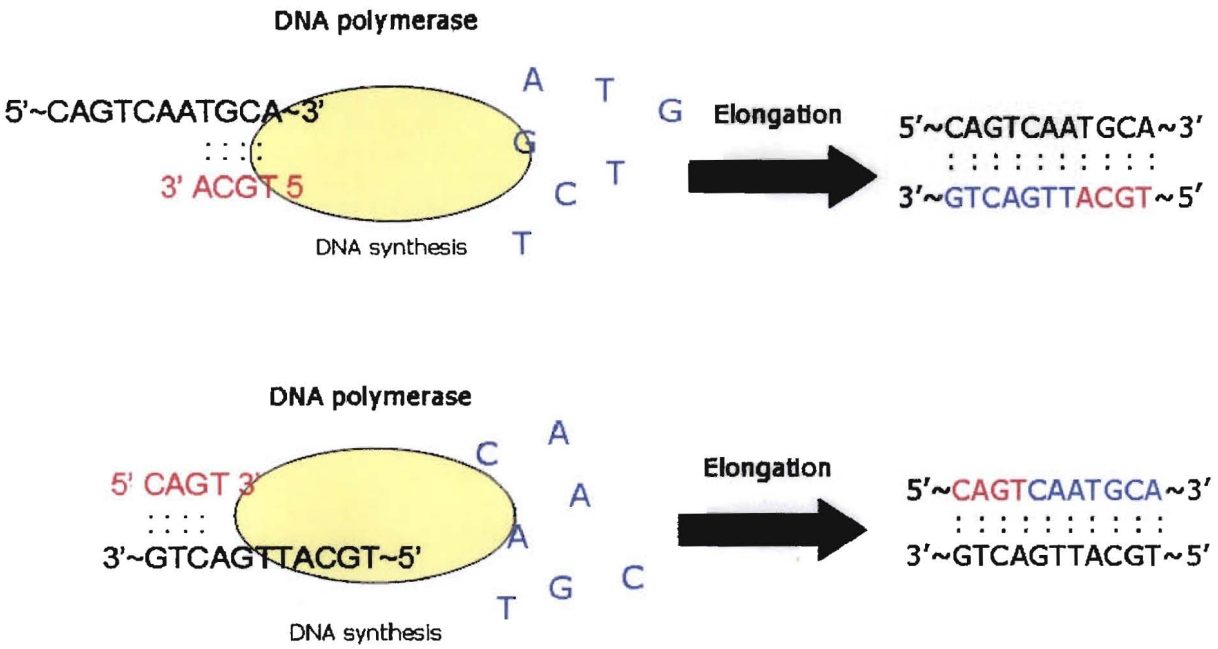

Figure 7. Once the primers anneal, the 3' end of each primer allows for the enzyme DNA polymerase to begin synthesis of new DNA.

The product of these reactions is a fragment of double-stranded DNA, which is as long as the sequence between the primer sequences in the template DNA.

These three basic steps of denaturation, annealing, and elongation make up one cycle. Cycles are repeated up to 40 times to generate a great number of DNA fragments.

Restriction enzymes cut DNA at very specific sequences: EcoRI, for example, recognizes only the sequence GAATTC. However, the sequence on either side of the EcoRI site can be anything. The PCR performed as a part of AFLP will allow us to only copy some of the fragments of DNA that were generated when the DNA of the whole cell is digested with restriction enzymes. This is done by selecting only those fragments with specific bases right after their restriction endonuclease site.

The procedure performed is outlined in Figure 8 (below). First, the DNA isolated from each sample is cut with the restriction enzymes EcoRI and MseI, both sticky end generators. Then, short DNA fragments called adapter molecules line up with restriction site sequences. These adapter molecules are added in the presence of an enzyme called DNA ligase. When an adapter molecule base pairs with its corresponding DNA fragment, there will be a gap in the DNA backbone between the two. DNA ligase seals this gap. The adapter fragments are used because their sequence is known. This allows for the PCR primers (which must have known sequences to be created in a lab) to adhere to the right spot to amplify the fragment.

Next, two PCR steps are performed; pre-selective amplification and selective amplification. All of the primers used in these steps base pair with the adapter fragment and restriction site sequences, which are known. They also contain extra base(s) selecting for only a fraction of the DNA fragments (Steps 3 and 4 of Figure 8). The pre-selective primers used contain an extra adenine nucleotide after the area where it base pairs with the DNA fragment; so only the fragments with an adenine directly after their restriction site could be amplified by DNA polymerase. Since one of the four possible nucleotides are selected for, only $^1/_4$ of the original fragments are amplified. Since every fragment will have two ends where primers anneal, each fragment has a $^1/_{16}$ chance of being amplified in the pre-selective amplification step. Twenty cycles of PCR were performed with the pre-selective primers.

For the selective amplification step, the primers used contain three extra nucleotides in from the restriction site sequence. Each primer of course has A as its first nucleotide, since the only fragments amplified in the previous step have an adenine on the 3′ end of their restriction sites. The next two nucleotides can be any that the researcher chooses. Also, the primers used in the selective amplification step have fluorescent dyes attached to them. This allows for laser detection once the reactions are done. In the selective amplification step, two more nucleotides are selected for: $^1/_{16} \times {}^1/_{16} = {}^1/_{256}$ of the fragments from the pre-selective amplification step are amplified. When the $^1/_{16}$ reduction in the number of fragments from the pre-selective amplification step is factored in, $^1/_{16} \times {}^1/_{16} \times {}^1/_{16} = {}^1/_{4096}$ of the original fragments created by the restriction digestion are amplified to detectable levels. The fragments that were not amplified will not be detected in the fingerprint, since they are not labeled with fluorescent dye.

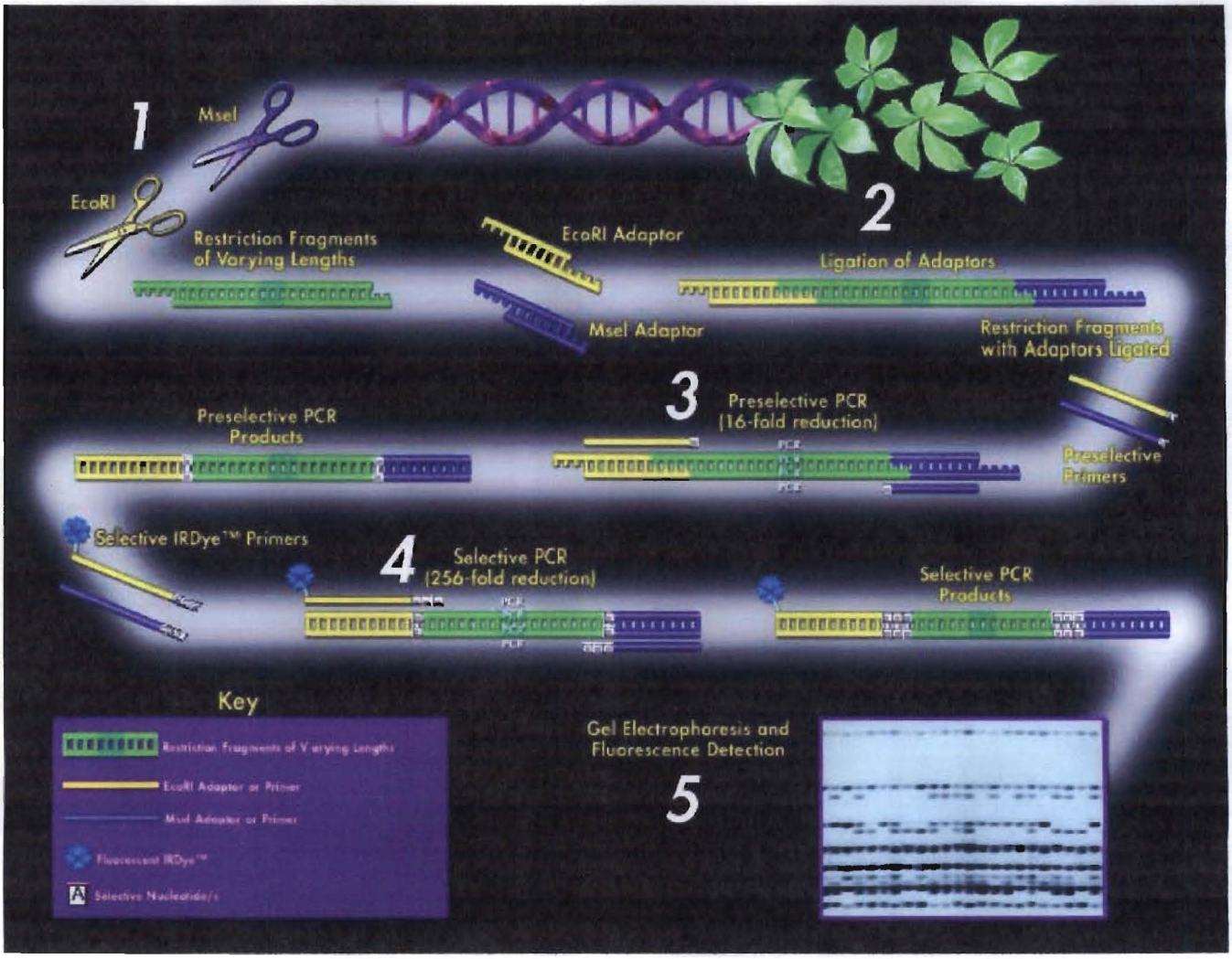

Figure 8. Diagram of AFLP procedure. In Step 1 the DNA is digested with the restriction enzymes EcoRI and MseI. The adapter molecules are ligated to the restriction fragments they base pair with in Step 2. Then in Step 3, pre-selective amplification is performed, using primers with one selective nucleotide. Step 4 shows the selective amplification step with primers containing the same first selective nucleotide as in the previous step, and two additional selective nucleotides. Step 5 is the gel electrophoresis, which separates the fragments based on size, and the detection of the bands using fluorescent dyes.

Once the amplification steps are completed, the reactions are loaded into a gel matrix and exposed to a current. This process is known as gel electrophoresis, and is a very common molecular biology tool. The phosphate groups on the backbone of every DNA molecule (Figure 2) give DNA a net negative charge, so when it is exposed to an electrical current, it will migrate towards the positive pole. The gel matrix, made of acrylamide, will make the DNA fragments migrate according to size. The larger fragments will not be as able to migrate through the gel matrix, and will be closer to the starting point in the gel (where the negative pole is). The smaller fragments can easily get through the gaps in the matrix, and will end up closer to the positive pole. Fragments that are the same size will end up migrating the same distance and create a visible band. In this way, the fragments generated by the restriction digestion and amplifications are separated to create a unique combination of bands for each sample. This banding pattern is used as a fingerprint of each

sample. To help gauge size of fragments, a standard is also loaded into the gel, which contains fragments of DNA with a known number of bases.

Importance of research:

In many species of fungi, the physical characteristics used to differentiate one species from another vary quite a bit within a species. This can make classification very complicated, and blurs the boundaries between one taxonomic group and another. There have been experiments involving DNA fingerprintings of fungi before, and many of them have found wide genetic variations. The genetic differences revealed by AFLP and other DNA fingerprinting techniques have been greater than the morphological differences in some cases. Reflecting on this data, there are so many questions to ask. So how much of the genome is important to the outward appearance of the fungus? Is high genomic variation present in species with high morphological variation? Is genomic variation positively correlated with distance between organisms as one would expect? The main question we pursued is, 'Does this variation occur in species that have unwavering physical characteristics?' The species we chose to study, *Polyporus squamosus*, has a very uniform outward appearance. Our hypothesis is that the DNA fingerprints will not have as wide a variety as other species because of the small differences between organisms' appearances. Since the morphology of this species is very distinct, and it exhibits almost identical physical characteristics in different areas of the country, there is probably very little genetic variation, since genes ultimately determine phenotype.

The mushrooms used in this study usually grow in clumps that arise from a single point. During collection of the samples, sometimes two mushrooms were collected from the same clump. These samples should be genetically identical to each other. If any differences in the fingerprints for these samples is found, then the genome will be more variable than the morphology of this species leads one to believe.

Also, these samples were collected from five different counties in east-central Indiana: Wayne, Fayette, Delaware, Henry, and Randolph. It is also our hypothesis that fingerprints will be very similar from samples collected in close proximity to each other, and that the farther away two samples are, the more different their fingerprints will be.

Reflection on experiences:

One of the lessons I have learned during this process is to always start early on things. If you know you will need a certain reagent, order it now so it is in by the time you need it (if possible). If you see a problem on the horizon, work to solve it now so that you aren't scrambling at the last minute. That occurred in this experiment with finding the right amount of DNA to add for each reaction. We want equal amounts of DNA added to each reaction, so that bands will be of equal strength when the results are seen. This is necessary because if one lane of the gel is darker than all the rest, we will know it is not because more DNA was added. The concentrations of DNA in each sample varied a lot, so something had to be done to either concentrate or dilute the samples as needed. It would have been helpful to have done this while we were getting the computer ready to go, so that we could just start right away when it was ready. Of course I had to balance taking classes and their work along with this research, not to mention applying to graduate schools and planning my wedding. In lab classes I have taken, there has been an emphasis on multi-tasking and balancing time to get all the experiments finished within the class time. This was certainly a time when I drew on that experience to accomplish my research in the least amount of time possible. 'Hindsight is always 20/20', as they always say, but in

hindsight it took me a while to effectively manage my time. But the only way to learn how to do some things is to try and fail; at least I knew how to do better by the end of this experience.

A hard part of this research was trying to weigh out 1 gram of frozen tissue, while keeping it frozen, but having to cut it up into pieces and see which combination of those pieces would get as close to 1 gram as possible! It is impossible to keep the tissue from thawing some, especially since the lab I used to weigh the tissue was on a different floor from where the next step of the procedure took place. Thawing probably resulted in a lower yield of DNA, since the tissue wasn't kept as fresh before the extraction procedure really began. Only experiences like this will make you think about the consequences of the decisions you make in a line of research. A single decision which may make sense logistically, like allowing tissue to thaw, can affect your whole experiment later on, in this case in the form of very poor yields of DNA. While performing an experiment, there can be hundreds of decisions like this to make, and one bad decision can make your experiment fruitless. It is impossible to always do the best thing for your experiment though, since many other factors need to be balanced to make the experiment do-able in a reasonable amount of time with a reasonable amount of resources. Only through out-of-class research can you truly appreciate the balancing act it takes to get an experiment done. Fortunately I was able to move quickly enough to prevent the DNA from serious degradation and obtain good yields of DNA in this situation, although I couldn't find that out until the end of each extraction procedure.

Another large problem was the new equipment used. The DNA analyzer, a new piece of equipment for the Biology Department, comes with its own computer and software, which my mentor Dr. Ruch learned how to use at a conference held by the manufacturer. The problem was the setup of everything on that computer, and then the programming of my specific experiment into the software, which took much longer than expected. This cut down on the time available to run the procedure using different reaction conditions, which are crucial towards optimizing the reactions for this specific species and method to get the best results possible.

The most frustrating part was seeing the results I got, which were enough to know that it was working for a few, but not all, of the samples. From all the specialized biotechnology classes I have taken, I knew of so many ways to optimize the reactions, and so many other places where we could change things to get the best possible results. The frustrating part was that I ran out of time to perform any alterations of the original procedure.

Despite the downfalls, this experience has been a most positive one overall. Doing experiments in a laboratory portion of a class are pretty different than doing independent research. The only way to understand this was to do independent research, and I wholly enjoyed it. My work with this project helped me decide to pursue a doctorate in biochemistry, and I will begin my studies for this at Indiana University Bloomington shortly after graduation. Although my project did not work out perfectly, I am so grateful to have had this experience, and I know it will help me enormously as I pursue my Ph.D. and during my scientific career.

DNA Fingerprinting via AFLP of the mushroom *Polyporus squamosus* Huds.:Fr.

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In this experiment, DNA from the mushroom *Polyporus squamosus* was fingerprinted using the technique amplified fragment length polymorphism (AFLP). The morphologic appearance of many fungi can vary a lot within a species, causing discontinuity over where species boundaries fall. *P. squamosus* is a species that has a well-defined appearance and morphology. The DNA fingerprints of this unambiguous species will help shed light on the relationship between genomic content, genomic diversity, and outward appearance.

In this experiment, the genomes of samples of the fungus *Polyporus squamosus* were analyzed using amplified restriction length polymorphism (AFLP) analysis. For many fungi, phenotypic characteristics vary widely between members of a species, making identification difficult and controversial and blurring the boundaries between taxonomic groups. DNA fingerprinting techniques, including AFLP, have revealed significant genetic differences between members of the same species (1-3), and in at least one case, the level of genomic diversity was higher than the level of morphological diversity (1). This raises many questions about the relationship between genomic content, genomic diversity, and the phenotype of an organism. AFLP uses the whole genome, both coding and non-coding, to make comparisons between closely-related individuals (species level), so this technique is a good way to explore this issue. The species *P. squamosus* was selected for this experiment because of its highly conserved phenotypic characteristics. The uniform phenotype of this species can be used to shed light on the influence of the genome as a whole on phenotype. It is because of this conservation of phenotype, along with the similar habitats the organisms were collected at, that we expect the fingerprints between all samples to be highly similar.

There are many things that can affect the genetic diversity of an organism. Differences in the local growing environment can contribute to differences in fungal genetic variation due to natural selection (1). This was addressed in our experiment by collecting only specimens growing on dead, hardwood trees. Also, most of the samples were collected in wooded areas. Genetic diversity of fungi has also been shown to be positively correlated with environmental stress (4). The effects of this a source of genetic variation in our sampling were minimized as samples were collected in the spring, where conditions are most favorable for the mushroom to sprout.

The AFLP method was first published by Vos et. al. (5) and was patented by Vos and Zabeau in 1993. The first step is the restriction digestion of the DNA with two restriction enzymes, creating thousands of fragments of the genome. Next, adapter pieces of DNA, which base pair with the sticky ends of the restriction digested fragments, are ligated onto the restriction fragments of the genome. The known sequence of the adapter fragments allow for PCR primers to amplify the genome fragments. The amplification of the genome fragments is selective: the sequence after the restriction site is not known, so PCR primers can contain 1, 2, or 3 bases after that site to select which fragments are amplified. This allows for only a small fraction ($^1/_{4096}$ if three selective

nucleotides are used) of the genome fragments generated by restriction digestion to be amplified to levels detectable by acrylamide gel electrophoresis (6).

AFLP as a fingerprinting method has many advantages: it is relatively cost-effective and easy to perform, and no known sequences are necessary. Also, the high degree of selectivity allows few errors compared to other fingerprinting methods, like random amplification of polymorphic DNA (RAPD). Very small amounts of DNA are used, and the procedure can be used on degraded DNA. Since the amplification sites are randomly selected, it is most likely some will be in highly variable areas of the genome, which can be used to reveal minute differences in closely related organisms. The main disadvantage of AFLP is that it only determines the presence or absence of a band- it cannot identify an allele or genotype (5-7). Since this experiment only dealt with the relatedness between members of the same species, AFLP was a useful method to choose.

EXPERIMENTAL PROCEDURES

P. squamosus samples were collected from 5 different counties in east-central Indiana (Table 1). All mushrooms used were mature and found growing on dead trees at each location. Some samples were taken from mushrooms growing from the same cluster. These are designated as _A and _B, and should be genetically identical. The remaining samples were taken from different individuals. Samples were taken from the anterior area of the cap, excluding the top scale layer and the bottom spore-forming layer, and then frozen at -80°C until use. DNA was extracted from approximately 1g of tissue using the Qiagen DNEasy Plant Maxi Kit (Qiagen, Cat. No. 69104). The DNA was collected off the column in two separate elutions made in PCR-grade water, and stored at -20°C. DNA was quantified using a spectrophotometer, and 100ng was used for AFLP analysis. AFLP was performed using the IRDye™ Fluorescent AFLP® Kit (LI-COR Biosciences, Cat. No. 4200-103) and Expand High FidelityPLUS Taq (Roche, Cat. No. 03300242001) using the selective primers M-CAA, E-AAC (700nm), and E-AGG (800nm). The banding pattern was analyzed using the 4300 NEN DNA Analyzer (Cat. No. 4300-04). 6.5% acrylamide gels were used. Samples were analyzed at both 700nm and 800 nm, producing two images per gel.

Table 1.

Sample	Reaction #	Location collected	Date collected	Notes
1	1	Lick Creek Summit, Wayne Co.	4/24/2004	
2	2	Duning Hoff Woods, Wayne Co.	4/24/2004	
3A	3	Mary Gray Nature Sanctuary, Fayette Co.	5/1/2004	Cap #2; Same organism as 3B
3B	4	Mary Gray Nature Sanctuary, Fayette Co.	5/1/2004	Cap #1; Same organism as 3A
4	5	Mary Gray Nature Sanctuary, Fayette Co.	5/1/2004	
5	6	Rt. 32, two miles west of Selma, north side of road along fence on stump; Delaware Co.	5/4/2004	
10	7	Red Tail Nature Preserve,	5/4/2004	

Table 1 con't.

		Delaware Co.		
11A	8	Red Tail Nature Preserve, Delaware Co.	5/4/2004	Cap #1; Same organism as 11B
11B	9	Red Tail Nature Preserve, Delaware Co.	5/4/2004	Cap #2; Same organism as 11A
13	10	IMI West Woods, Henry Co.	5/2/2005	
14	11	Yuhas Woods, Randolph Co.	5/3/2005	
15	12	Yuhas Woods, Randolph Co.	5/3/2005	
16	13	Lick Creek Summit, Wayne Co.	5/4/2005	
17	14	Lick Creek Summit, Wayne Co.	5/4/2005	
18A	15	Lick Creek Summit, Wayne Co.	5/4/2005	Cap #1; Same organism as 18B
18B	16	Lick Creek Summit, Wayne Co.	5/4/2005	Cap #2; Same organism as 18A
19	17	Lick Creek Summit, Wayne Co.	5/4/2005	
20A	18	IMI Central Woods, Henry Co.	5/12/2005	Cap #1; Same organism as 20B
20B	19	IMI Central Woods, Henry Co.	5/12/2005	Cap #2; Same organism as 20A
21A	20	Yuhas Woods, Randolph Co.	5/17/2005	Cap #1; Same organism as 21B.
21B	21	Yuhas Woods, Randolph Co.	5/17/2005	Cap #2; Same organism as 21A.
22A	22	Yuhas Woods, Randolph Co.	5/21/2005	Cap #2; Same organism as 22B
22B	23	Yuhas Woods, Randolph Co.	5/21/2005	Cap #1; Same organism as 22A

Table 1. Locations and dates of samples collected in east-central Indiana counties. Samples designated as '_A' and '_B' were taken from the same cluster of mushrooms. Samples without a letter were taken from different clusters.

RESULTS

The concentration of DNA isolated from each sample can be found in Table 2. The concentrations range from 0.015 to 0.135 µg/µL, and were diluted 5X to obtain 100ng for use in the AFLP procedure in a workable volume.

Continued on page 19

Table 2.

Sample	Reaction #	A_{260}	Conc. (µg/mL)	Conc. (µg/µL)	100ng = xµL	100ng = xµL in 5x diluted sample
1	1	0.027	135	0.135	1.35	6.75
2	2	0.018	90	0.09	0.9	4.5
3A	3	0.012	60	0.06	0.6	3
3B	4	0.019	95	0.095	0.95	4.75
4	5	0.003	15	0.015	0.15	0.75
5	6	0.015	75	0.075	0.75	3.75
10	7	0.003	15	0.015	0.15	0.75
11A	8	0.005	25	0.025	0.25	1.25
11B	9	0.003	15	0.015	0.15	0.75
13	10	0.008	40	0.04	0.4	2
14	11	0.011	55	0.055	0.55	2.75
15	12	0.004	20	0.02	0.2	1
16	13	0.01	50	0.05	0.5	2.5
17	14	0.017	85	0.085	0.85	4.25
18A	15	0.012	60	0.06	0.6	3
18B	16	0.006	30	0.03	0.3	1.5
19	17	0.01	50	0.05	0.5	2.5
20A	18	0.014	70	0.07	0.7	3.5
20B	19	0.012	60	0.06	0.6	3
21A	20	0.003	15	0.015	0.15	0.75
21B	21	0.009	45	0.045	0.45	2.25
22A	22	0.024	120	0.12	1.2	6
22B	23	0.008	40	0.04	0.4	2

Table 2. The original concentrations of DNA from each sample ranged from 0.015 to 0.135 µg/µL. Concentration of each DNA sample was found using a spectrophotometer and measuring the absorbance at 260nm.

Figure 1.

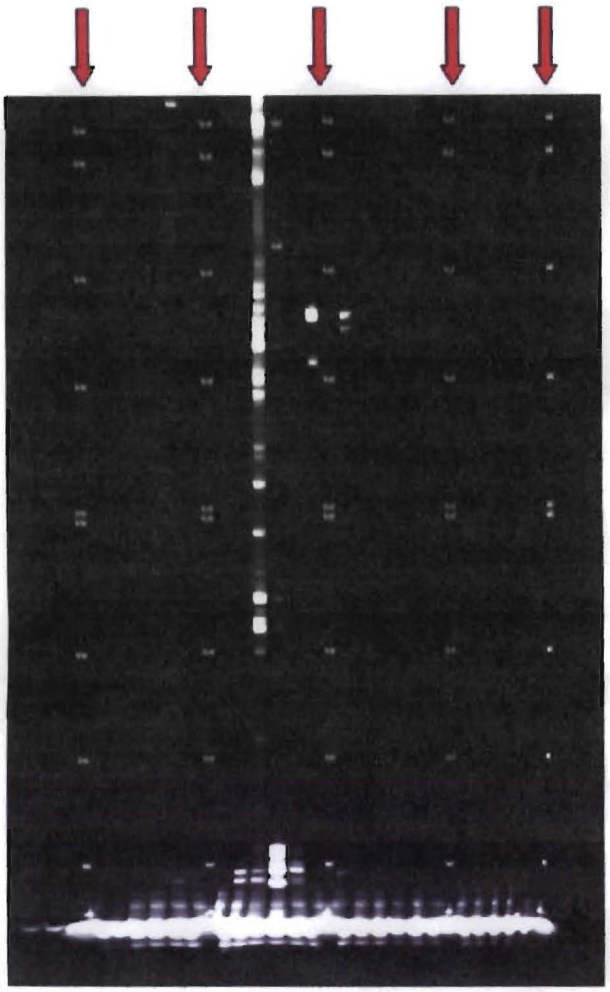
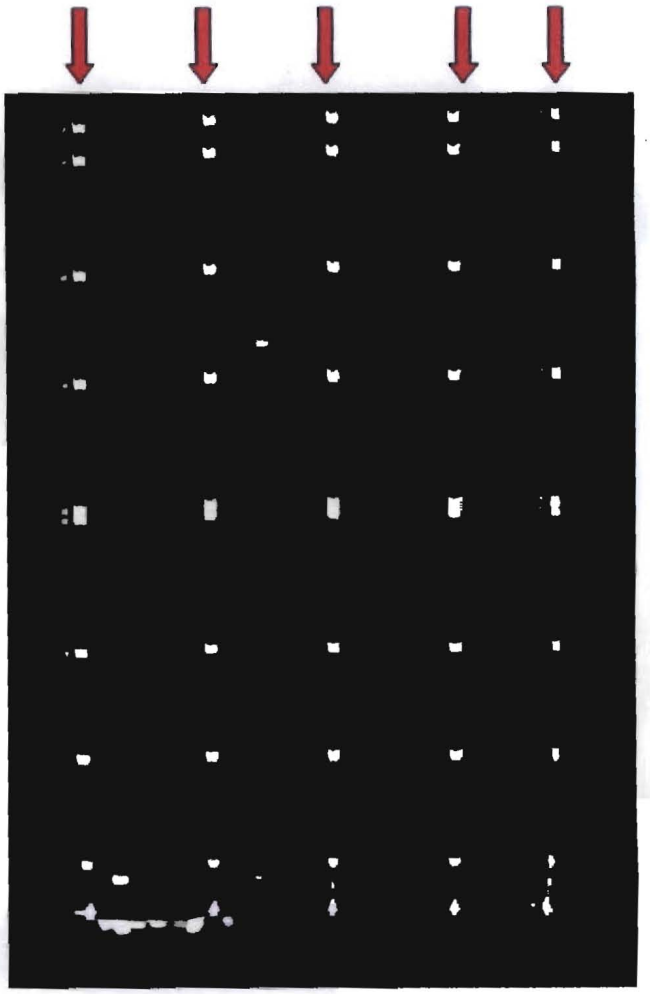


Figure 2.



Figures 1 and 2. The results of first AFLP procedure shows only one sample with significant amplification. The 700nm (left) and 800nm (right) results for the first AFLP procedure are shown above. The red arrows indicate lanes with standard molecular weight markers. The only sample to give more than three bands is 11B, in lane 11. No analysis was performed.

Figure 3.

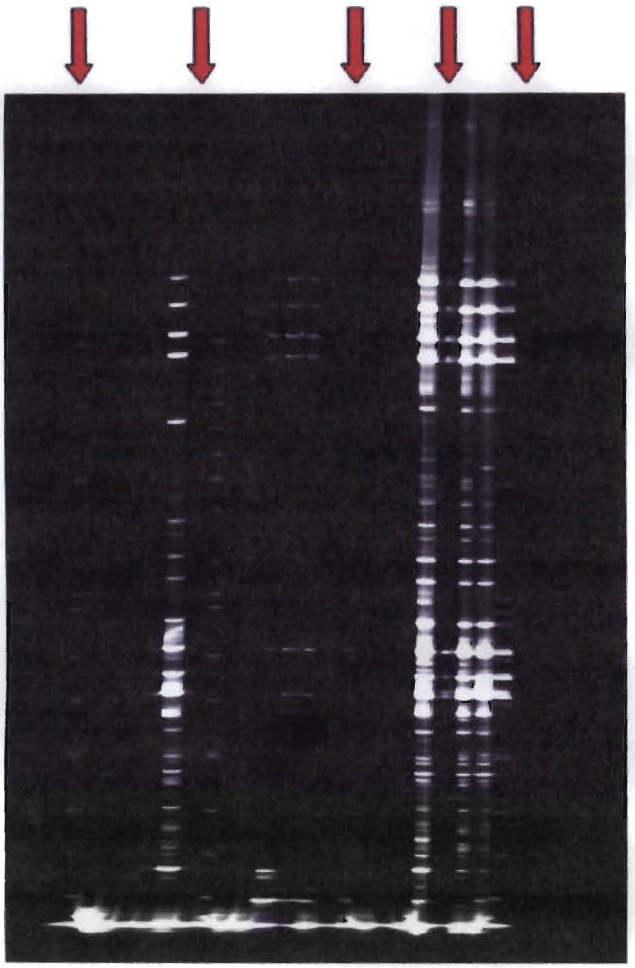
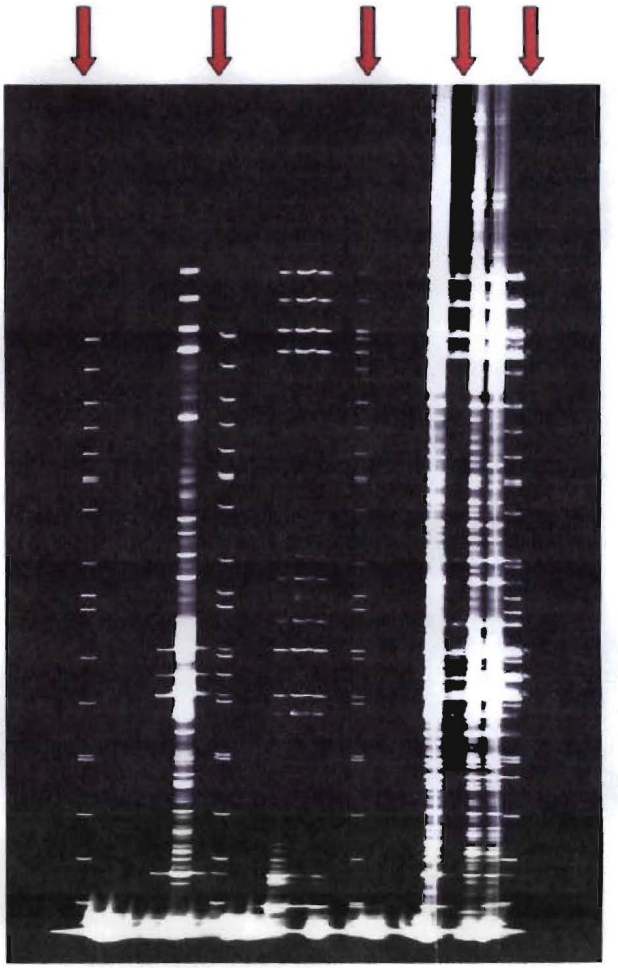


Figure 4.



Figures 3 and 4. Amplification was detected for nine samples during the second round of AFLP. Only samples 1- 20B were used in this AFLP procedure. The 700nm (left) and 800nm (right) results are shown above. The red arrows indicate lanes with standard molecular weight markers. Samples 4, 11A, 11B, 13, 14, 18B, 19, 20A, and 20B show significant amplification. The rest of the samples show 4 bands or less. Due to the smearing present, and the lack of bands for many samples, no analysis was performed.

Figure 5.

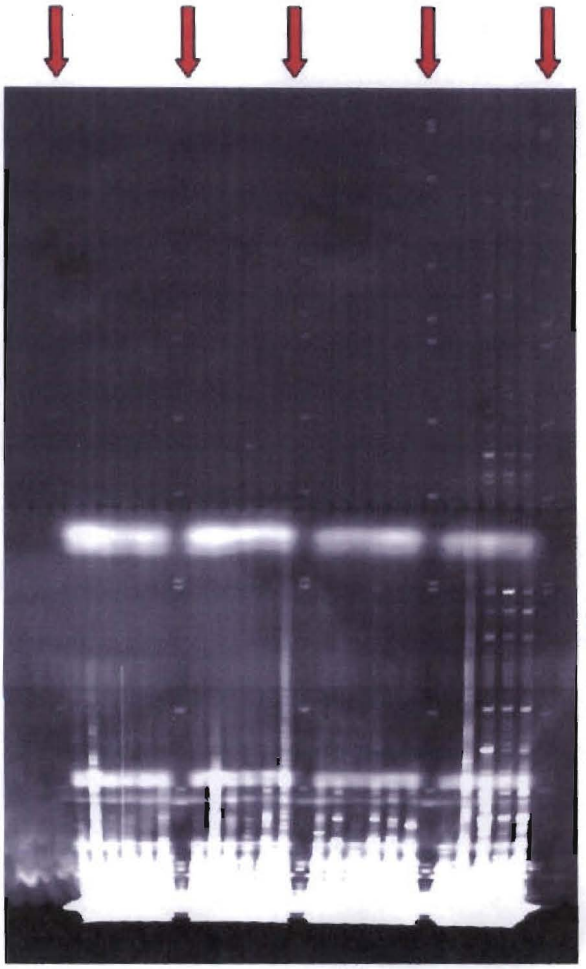
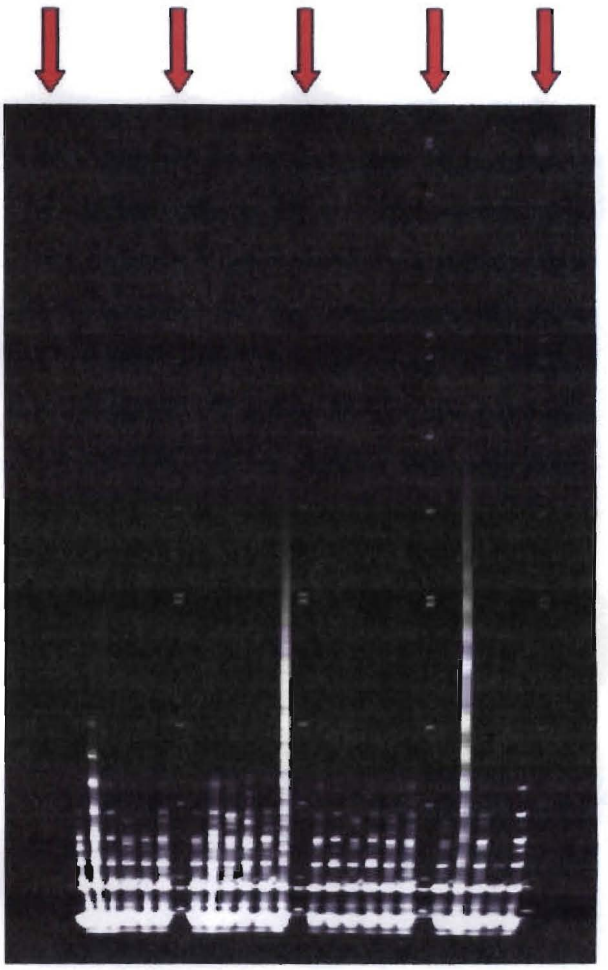


Figure 6.



Figures 5 and 6. Little amplification took place during the third round of AFLP. The 700nm (left) and 800nm (right) results are shown above. The red arrows indicate lanes with standard molecular weight markers. This experiment shows differences between the 700 and 800 pictures. The 700nm detected three samples with good amplification (21B, 22A, and 22B), which do not appear in the 800 picture. The 800 also shows more low molecular weight bands than the 700 picture.

The AFLP procedure was performed three times, twice with all samples, and once with samples 1- 20B. The results, with both the 700nm and 800nm images, are in Figures 1-6. At no time did all samples produce a fingerprint in the same gel. The same primers were used for each reaction (see Experimental Procedures). All samples, when run on the gel, showed a few, low molecular weight bands, but only a few samples had more than 4 bands. This could indicate a problem occurred in the amplification steps for most of the samples. It could also indicate poor quality DNA was used in the reaction.

In the third AFLP gel, there are some differences between the 700nm and 800nm images. The 700nm image shows that amplification has occurred for the last three samples on the gel, while these bands are not present on the 800nm image. Also, the 800 image shows more low-molecular weight bands.

DISCUSSION

The imperfect results of this experiment are most likely the cause of problems in the PCR amplification steps. There are a great number of things that could be done to combat this. First of all, different primers could have been used. Different concentrations of the primers could also have been used and the results compared to optimize the reaction. A proteinase digestion could also be performed, to remove any protein contaminants which may be interfering with the reaction. Also, re-extraction of DNA from samples should be performed for samples which show no bands on any of the three gels.

When viable fingerprints have been obtained for every sample, there are many new directions in which to proceed. A very appropriate experiment would perform AFLP on cDNA isolated from each sample. With a cDNA fingerprint, it is possible to determine which bands from the whole genome fingerprint involve coding DNA. In this way, the frequency of polymorphisms of coding and non-coding DNA can be determined for each sample. It will also be possible to investigate whether non-coding DNA contains more polymorphisms than the coding DNA.

A lot of questions still remain regarding evolutionary biology. No one knows how much the non-coding DNA of an organism affects evolution, or how much of it must remain unchanged for maximum fitness. No one has yet found a way to determine whether diversity in non-coding DNA is important to cellular functions. The experiments attempting to find answers to these questions are on the forefront of a new field in biology. AFLP is a method that measures both coding and non-coding DNA, since fragments from random points in the genome are used to create a fingerprint. This method and others are beginning to work on this knotted question, but different techniques are needed to determine the effect of non-coding DNA on an organism's diversity, fitness, and morphology.

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